Remarks

A. Objection Under 35 U.S.C. § 132(a)

Paragraph [0001] of the specification has been amended to delete the phrase "incorporated by reference herein in its entirety." Accordingly, it is respectfully requested that the Examiner withdraw the new matter objection.

B. Rejection Under 35 U.S.C. § 112, second paragraph

Applicant has amended claims 26 – 31 to correct the dependency of such claims. In addition, the phrase "effective amount" has been deleted from claims 28 – 30. The Examiner is respectfully requested to withdraw this rejection.

C. Rejection Under 35 U.S.C. § 102(b)

The Examiner rejected Claims 23, 26 – 28, and 31 under 35 U.S.C. § 102(b) as anticipated by Tokoro. Applicant respectfully asserts that the amended claims of the present application are not anticipated by Tokoro. Specifically, the claims of the present application are not anticipated by Tokoro because Tokoro does not teach the production of a transfer factor.

Despite the fact that transfer factors have been studied for over 50 years and over 3,000 articles have been published in peer-reviewed scientific journals regarding transfer factors, Tokoro lacks any discussion of transfer factors. Tokoro does not teach that transfer factor is present in its hen eggs but, rather, that a "transfer factor-like component" is present in the eggs. By using the term "like" in the description of the "transfer factor-like component", Tokoro teaches that something other than transfer factor was actually present in the eggs and, thus, Tokoro cannot anticipate the present invention.

"Transfer factor-like" is a term of art that indicates a substance which has transfer factor-like activity, but is not actually transfer factor. The term "transfer factor-like" is described in Dunnick, W., *et al.*, "*Lack of Antigen Fragments in Guinea Pig Transfer Factor-like Activity*," Clin. Immunol. and Immunopathol. 17:55-65 (1980) (copy enclosed). Dunnick refers to the substance evaluated therein, which has transfer factor-like activity, as "TFLA". Based on the experiments described in the reference, Dunnick concludes that superantigenicity, a characteristic of transfer factor (p. 55), "cannot explain the activity of TFLA." (Dunnick, p. 65). Dunnick states that "[w]hereas transfer factor and TFLA are structurally similar . . . and the tests for the two are related, no direct relationship has been established between TFLA and *in vivo* transfers of cellular immunity." (Dunnick, pg. 65). It has long been known that transfer factors are capable of transferring cellular immunity *in vivo*. Thus, it is clear that transfer factor is <u>different</u> from the "transfer factor-like component" of Tokoro and that a "transfer factor-like component" will not necessarily transfer immunity. Therefore, Tokoro cannot anticipate the present invention.

In its disclosure, Tokoro even suggests that its "transfer factor-like component" is not <u>actually</u> transfer factor. The Tokoro reference states, "the immunological functions of the transfer factor-like component . . . are not known." (Tokoro, col. 7, lines 44-47). In contrast, the immunological functions of transfer factor are and have been known for years. Additionally, Tokoro states that "[t]here is a possibility that a part of the transfer factor-like component is the same as the food factor described in [U.S. Pat. No. 4,402,938]." (Tokoro, col. 7, lines 51-54). If, as Tokoro asserts, there is a possibility that its transfer factor-like component is the same as the food factor described in U.S.

Pat. No. 4,402,938, it cannot also be transfer factor. This statement is further evidence that even Tokoro does not believe that its "transfer factor-like component" is truly transfer factor. If Tokoro believed its "transfer factor-like component" were transfer factor, the reference would reflect this.

MPEP § 2131 provides that a claim is anticipated only if each and every element as set forth in the claim is described in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). "The identical invention must be shown in complete detail as contained in the . . . claim." *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989). Because Tokoro does not teach every element of the claim, namely the production of transfer factor, it cannot anticipate the present invention and a *prima facie* case of anticipation has not been satisfied.

The Examiner asserts that "the name used to describe the crude preparation obtained by the method of Tokoro is irrelevant because it contains all molecules less than 10,000 mw, including transfer factor." Merely because the emulsion is filtered to contain "all molecules less than 10,000 mw" and a "transfer factor-like component" was recovered from that emulsion, and transfer factor has a molecular weight of less than 10,000, does not mean that the "transfer factor-like component" is transfer factor. For example, both ethanol and water have a molecular weight that is less than 50. Ethanol and water are not the same substance merely because they both have molecular weights of less than 50. Similarly, the method of Tokoro does not necessarily result in a product that contains transfer factor merely because Tokoro uses a filtration step that separates particles having a molecular weight of less than 10,000. As discussed

above, the term "transfer factor-like component" has a specific meaning in the art and it is clear that Tokoro does not believe its "transfer factor-like component" is transfer factor. Accordingly, a *prima facie* case of anticipation has not been satisfied.

D. Rejection Under 35 U.S.C. § 103(a)

The Examiner rejected Claims 23 and 26 – 31 under 35 U.S.C. § 103(a) as obvious over Tokoro in view of Anderson. The above arguments apply equally to the obviousness rejection and should be deemed to be repeated here. The combination of references fails to teach or suggest all elements of the claims. MPEP § 2142 - § 2143 requires that in order to make a *prima facie* case of obviousness, the cited prior art reference must teach or suggest all the claim limitations. Anderson is wholly unrelated to transfer factors. As discussed above, Tokoro fails to teach the production of a transfer factor. Because the combination of Tokoro and Anderson do not teach or suggest a "process for producing transfer factor," the references cannot satisfy a *prima facie* case of obviousness.

In addition, Tokoro teaches away from the claims of the present invention by teaching that, unlike transfer factor, its "transfer factor-like component" has unknown immunological properties. Tokoro also asserts that, unlike transfer factor, part of the "transfer factor-like component" may be the same as a food factor. Teaching away is the antithesis of art suggesting that the person of ordinary skill go in the claimed direction. *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988). Thus, it is clear that upon reading Tokoro, one skilled in the art would not be led to use the method of present invention to produce transfer factor. Accordingly, the Examiner has not satisfied a *prima facie* case of obviousness in this case.

In summary, Applicant submits that the claims and specification are now in condition for allowance. It is respectfully submitted that claims 23-31 are patentably distinct over the references cited by the Examiner and meet all other statutory requirements. Therefore, reconsideration of the rejections in the Office Action is respectfully requested. The Examiner is invited to telephone the undersigned should any issues remain after the consideration of this response.

Please charge any additional fees that may be required to Deposit Account No. 50-2548.

Respectfully requested,

NELSON MULLINS RILEY & SCARBOROUGH

July 25, 2006
Date

Nichole Andrighetti
Registration No. 56,508
Meridian, Suite 1700
1320 Main St.
Columbia, SC 29201
Phone: (864) 250-2292
Fax: (803) 255-9831

CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY 17, 55–65 (1980)

Lack of Antigen Fragments in Guinea Pig Transfer Factor-like Activity

W. DUNNICK,[1] DENIS R. BURGER, AND ARTHUR A. VANDENBARK

Surgical Research Laboratory, Veterans Administration Hospital, and Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oregon 97207

Received December 12, 1979

Transfer factor-like activities (TFLA) were prepared from guinea pigs immunized to ovalbumin (OA) or to glutamic acid–tyrosine copolymers (GT). Major steps in the partial purification of these factors included gel filtration, alkaline phosphatase treatment, and DEAE-cellulose chromatography. The TFLA were detected by antigen-dependent migration inhibition of nonimmune peritoneal exudate cells. Nonimmune cells incubated with OA TFLA and OA were inhibited from migrating, cells incubated with OA TFLA and GT were not. Nonimmune cells incubated with GT TFLA and either GT or OA were inhibited from migrating, although the inhibition in the presence of OA was small compared to the inhibition of cells incubated with OA TFLA and OA. "Superantigenicity" did not seem to be an explanation for the activity of these factors, since immunization of donors with radioiodinated antigens did not result in significant incorporation of radiolabel into fractions active in the migration inhibition test.

INTRODUCTION

A simple hypothesis which explains both the activity (1) and the biochemical composition (2–4) of transfer factor is that it is a "superantigen." Superantigens are antigen fragments, often associated with nucleic acid, and seem to be unusually immunogenic (5–7). Transfer factor cannot be conventional antigen, as it is dialyzable (1), and human transfer factor for tuberculin does not seem to bind to anti-tuberculin antisera (8). In addition, passage of a guinea pig transfer factor through an antidinitrophenyl column does not affect its ability to transfer contact sensitivity to dinitrofluorobenzene (9). Nevertheless, it is possible that the antigenic fragments in transfer factor might not bind to antibodies (because the determinants are masked, or are different for T cells and for antibody, or are too small), and yet transfer cellular immunity.

We have approached the problem of antigen fragments in transfer factor using an *in vitro* system. If incubated with both antigen and a low molecular weight factor from immune guinea pig leukocytes, nonimmune guinea pig leukocytes respond by migration inhibition and thymidine uptake (10). These antigen-dependent responses are specific for the donor immunities (11). An advantage of this system is that the active moiety can be purified away from most cellular components by a combination of gel filtration, enzyme treatment, and ion exchange chromatography. As a test for antigen fragments in this transfer factor-like activity (TFLA), we have immunized guinea pigs with radiolabeled antigen, and

[1] Address correspondence and reprint requests to W. Dunnick, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England.

0090-1229/80/090055-11$01.00/0

monitored the purified factors for radioactivity. We have also tested the activity and specificity of these purified factors.

MATERIALS AND METHODS

TFLA preparation. TFLA were purified through the DEAE-cellulose chromatography step as previously described (11). Strain 13 guinea pigs (Happy Mouse Ranch, Port Angeles, Wash.) were immunized with ovalbumin (OA, 100 μg) or glutamic acid–tyrosine copolymer (GT, 1000 μg) in complete Freund's adjuvant by injection of 0.1 ml per footpad (10).

Donors of OA TFLA were injected intraperitoneally with mineral oil 9 days after immunization and donors of GT TFLA 25 to 28 days after immunization. Three days later peritoneal exudate, spleen, and lymph node cell suspensions were prepared (10), and cells were lysed by three freeze–thaw cycles. The cell lysates were pooled and centrifuged at 20,000*g* at 4°C for 40 min, and the resulting supernatant fluid was applied to a 2.5 × 98-cm Sephadex G-25 (fine) column. The material was eluted with 50 m*M* ammonium bicarbonate at 30 ml/hr at 4°C, and 10-ml fractions were collected. Peak 4a (Fig. 1A) was pooled and lyophilized. The lyophilized material was dissolved in 2 ml 10 m*M* Tris, 1 m*M* magnesium chloride, pH 7.8, at 22°C. Alkaline phosphatase (Sigma, P4252) was added to a final concentration of 0.3 enzyme units to one 260-nm absorbance unit of peak 4a material. The mixture was incubated at 37°C for 30 min and then applied to a 1 × 28-cm Sephadex G-25 (fine) column. The material was eluted with 50 m*M* ammonium bicarbonate at 5 ml/hr at 4°C, and 2-ml fractions were collected. Peak 4a (Fig. 1B) was pooled and applied to a 0.6 × 4-cm DEAE-cellulose column. The material was eluted with 50 m*M* ammonium bicarbonate at 12 ml/hr at 4°C until the initial absorbance returned to baseline, and then was eluted with an 80-ml linear gradient increasing from 50 to 600 m*M* ammonium bicarbonate. Two-milliliter fractions were collected. Various fractions were pooled and tested for activity in the migration inhibition test.

Antigen radiolabeling. OA and GT were dialyzed overnight against 500 vol 50 m*M* sodium phosphate, pH 7.4, at 4°C. To 1280 μg (200 μl) GT or 120 μg (10 μl) OA was added 200 μCi sodium [^{125}I]iodide (New England Nuclear, NEZ-033H) in 10 μl 250 m*M* sodium phosphate. The solution was mixed, and 50 μg chloramine T in 10 μl 50 m*M* sodium phosphate was added. The solution was mixed 60 sec, diluted to 300 μl with 50 m*M* sodium phosphate, and dialyzed overnight against 50 m*M* sodium phosphate at 4°C. The contents of the dialysis bag were emulsified with 0.3 ml complete Freund's adjuvant, and injected into a Strain 13 guinea pig, ca. 0.15 ml per footpad. TFLA was prepared from these guinea pigs as described above. An aliquot of the radiolabeled antigen was stored and counted on the same day the TFLA was recovered from the initial Sephadex G-25 column.

The migration inhibition test. The migration inhibition test was performed essentially as described elsewhere (10). Oil-induced peritoneal exudate cells from a nonimmune Strain 13 guinea pig were incubated with combinations of TFLA and antigen in 16 × 100-mm siliconized glass culture tubes for 15 to 16 hr in a humidified incubator at 37°C. Each tube contained a final volume of 0.4 ml RPMI 1640 with 25 m*M* Hepes and 5% (v/v) autologous serum; an equal aliquot of cells

(ca. 5×10^6); 50 μg/ml OA, 10 μg/ml GT, or no antigen; and TFLA. TFLA was typically tested at 10^{18}, 10^{19}, and 10^{20} dilutions. Dilutions were made by serial 10-fold, 100-fold, or 1000-fold steps, using a clean pipet for each dilution step. After overnight incubation, the cells were recovered by centrifugation at 250*g*, resuspended in a small amount of the supernatant fluid, and loaded into capillary tubes. After centrifugation (80*g*) the capillary tubes were cut at the cell–liquid interface and placed in migration chambers (Cooke, 308). The chambers contained a volume of 0.4 ml RMPI with 25 m*M* Hepes and 15% (v/v) autologous serum, and antigens corresponding to those in the preincubation tubes. After 24 hr the migration areas were evaluated without knowledge of the treatment of the cells. Inhibition was calculated as follows:

$$100 \times \left[1 - \left(\frac{\text{migration area of cells treated with TFLA and antigen}}{\text{migration area of cells treated with TFLA only}} \right) \right].$$

Fluorescamine reaction. One-microliter samples for determination of primary amines were diluted with 25 μl 6 *N* hydrochloric acid (fresh), and hydrolyzed 18 hr at 160°C in sealed glass ampules. The acid was removed under vacuum with gentle heating. Samples were redissolved in 1.2 ml 10 m*M* sodium borate (pH 9.0) and pH was adjusted to 9 with 1 μl 1 *M* sodium hydroxide. Four-tenths milliliter fluorescamine in acetone (200 μg/ml) was added to each sample with vigorous stirring. Fluorimetry was accomplished by exciting samples at 390 nm and reading relative fluorescence at 485 nm. Glycine was used as a standard, and yielded linear fluorescence from 125 to 2000 pmol. Hydrochloric acid reagent blanks yielded 160 pmol of primary amine and were subtracted from all samples. TFLA samples (0.01 guinea pig equivalents, 0.001 absorbance units at 260 nm) yielded raw values of 300 to 500 pmol.

Amino acid analysis. A thrice-lyophilized sample was hydrolyzed, trifluoroacylated, and esterified with *n*-butyl alcohol. Gas chromatography (gc) was employed for amino acid analysis of the trifluoroacetyl-*n*-butyl esters (16). This analysis was carried out using a Varian 2700 gas chromatograph equipped with a four-channel DuPont MSID accessory for specific ion monitor analyses. The 1.8-m glass gc column (i.d. 2 mm) was packed with 3% OV-210 on Gas-Chrom Q 80/100 mesh. The precolumn between the injection port and cold spot was packed with 1.5% OV-101 on Gas-Chrom Q 100/120 mesh.

Data analysis. TFLA displays a dose optimum above and below which responses are not observed. Doses removed threefold from the optimal yield ca. 50% of the optimal activity, those removed 10-fold little or no activity (10, 16). The optimal dose can shift slightly from one experiment to another. We have chosen to analyze such data by reporting the greatest migration inhibition of the two or three dilutions tested. A slight bias is introduced by this selection procedure; this bias is partially corrected by comparing mean percentage inhibitions by unpaired tests, even though the data are naturally paired within each experiment as plus and minus TFLA treatments.

For analysis of specificity (Table 4), plus TFLA migration indices were first divided by no TFLA migration indices to correct for differences between antigens

in the absence of factors. Then, specificity was tested by reporting the results, for both antigens tested, of the TFLA dilution that led to the greatest inhibition for either antigen, regardless of the immunization of the donor (discussed in detail in Ref. (10)). The values for the two antigens were compared by paired t tests in a consecutive series of experiments.

Experimental migration areas were compared to no TFLA or no antigen controls by one-tailed t tests. In general, inhibitions less than 10% were rarely significant, those 10 to 15% occasionally significant, those 15 to 20% often significant, and those greater than 20% almost always significant.

RESULTS

Donor Immune Responses

Twelve days after immunization donors of OA TFLA show 15- to 25-mm induration when challenged intradermally with 5 μg OA. Peritoneal exudate cells taken at the time of TFLA preparation are inhibited from migrating 50 to 60% by OA, but not at all by GT. Immune responsiveness to GT seems to be weaker and take longer to develop (17). Three to four weeks after immunization, GT TFLA donors show 8- to 12-mm induration when challenged with 5 μg GT; peritoneal exudate cells are inhibited from migrating 30% by GT and 10 to 15% by OA. The inhibition in the presence of OA was often not significant, but was reproducible from one animal to another.

Preparation of Partially Purified TFLA

Absorbance profiles obtained on fractionation of OA immune leukocyte lysates were similar to those previously published for OA and bovine γ globulin leukocyte lysates (11). The absorbance peak eluting slightly after one column volume from Sephadex G-25 (peak 4a, Fig. 1A) led to OA-dependent migration inhibition (data not shown). This active fraction yielded two absorbance peaks after alkaline phosphatase treatment and rechromatography on Sephadex G-25 (Fig. 1B). Of the material remaining in peak 4a, most did not bind to DEAE-cellulose; a small portion bound and was eluted with a salt gradient.

Fractionation of GT immune leukocyte lysates yielded a similar absorbance pattern. However, GT lysates showed little absorbance in DEAE-cellulose fraction 2 compared to OA lysates (Figs. 1C and 1D).

DEAE-cellulose fractions 2 to 6 from GT immune cells were evaluated in the migration inhibition test (Table 1). GT alone did not inhibit the migration of these nonimmune cells, 205 compared to 203. Nor did fractions 2 to 6 alone significantly inhibit the migration of the cells. Fraction 3 at a 10^{19} dilution and fraction at a 10^{18} dilution led to significant inhibition in the presence of GT, whether compared to no TFLA or no antigen controls. These results reemphasize the dose–response characteristics of TFLA previously noted (10, 16). Dilutions of TFLA 10-fold too great (10^{19} for fraction 5) or 10-fold too small (10^{18} for fraction 3) reduced or eliminated the ability to induce migration inhibition responses. Fractions 2, 4, and 6 did not lead to migration inhibition at either 10^{18} or 10^{19} dilutions.

TFLA activity from GT immune leukocyte lysates was consistently focussed in DEAE-cellulose fractions 3 (titer of 10^{19}) and 5 (titer of 10^{18}, Fig. 2). Fractions 2

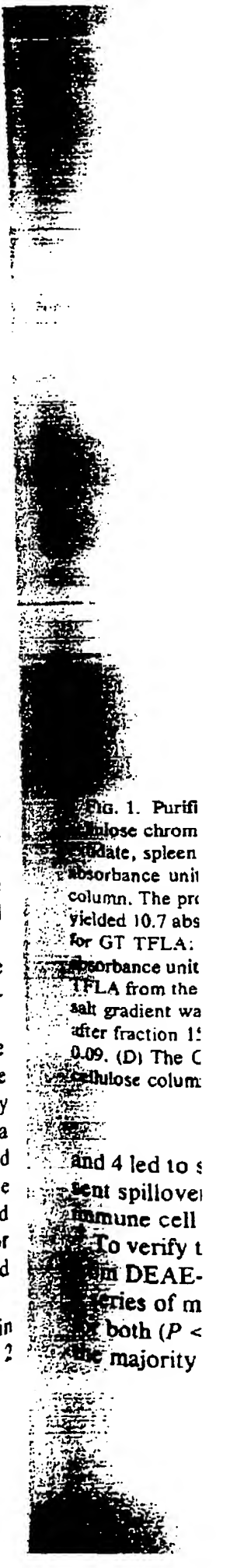

FIG. 1. Purifi
lose chrom
date, spleen
absorbance unit
column. The pr
yielded 10.7 abs
for GT TFLA:
absorbance unit
TFLA from the
salt gradient wa
after fraction 1
0.09. (D) The C
cellulose colum

and 4 led to s
ent spillove
mmune cell
To verify t
m DEAE-
eries of m
both ($P <$
majority

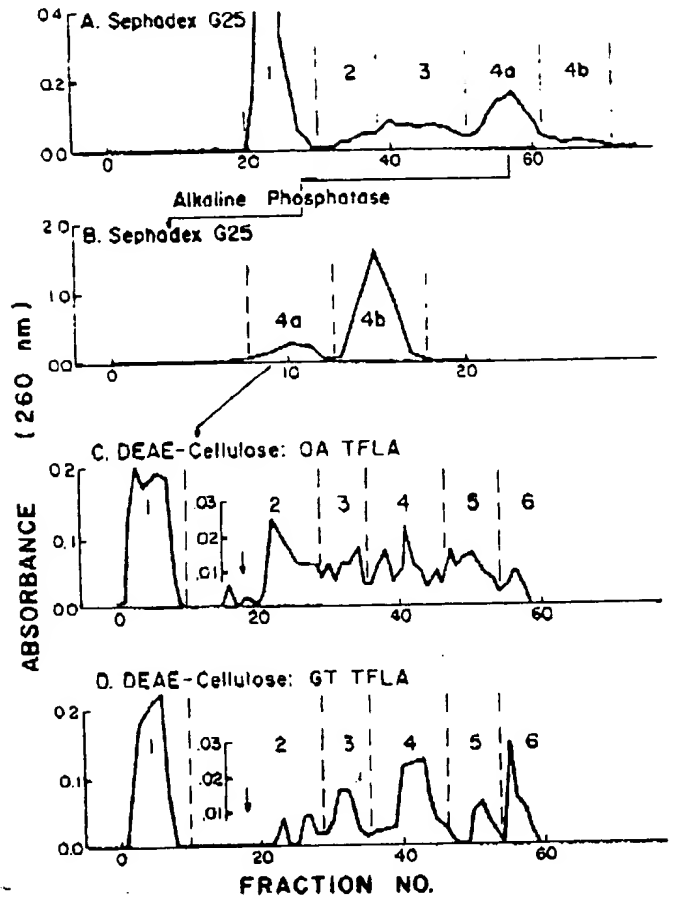

FIG. 1. Purification of OA and GT TFLA. OA and GT TFLAs were purified through the DEAE-cellulose chromatography step as described under Materials and Methods. (A) Combined peritoneal exudate, spleen, and lymph node cell lysates from a single guinea pig immunized to OA (total lysate absorbance units, 260 nm: 50.7) or GT (absorbance units, 56.8) were applied to a Sephadex G-25 column. The profile shown is for GT TFLA, although the factors gave similar profiles. The OA factor yielded 10.7 absorbance units in fraction 4a; the GT factor, 10.3. (B) The absorbance profile shown is for GT TFLA; again, the factors yielded similar profiles after alkaline phosphatase treatment. 2.4 absorbance units remained in Sephadex G-25 fraction 4a for OA TFLA, 2.0 for GT TFLA. (C) The OA TFLA from the second Sephadex G-25 column was applied directly to a DEAE-cellulose column; the salt gradient was initiated at fraction 18 (arrow). Note that the absorbance scale is expanded 20-fold after fraction 15 in both (C) and (D). Fraction 2 included 0.15 absorbance units, fraction 3 included 0.09. (D) The GT TFLA from the second Sephadex G-25 column was applied directly to a DEAE-cellulose column. Fraction 2 included 0.04 absorbance units, fraction 3 included 0.11.

and 4 led to small (not significant) inhibitions; these small inhibitions could represent spillover from fraction 3. Fraction 1 is not active in OA or bovine γ globulin immune cell lysates (11); it was not tested in these experiments.

To verify that both OA and GT TFLA elute in the early part of the salt gradient from DEAE-cellulose, we tested OA and GT DEAE-cellulose fractions 2 and 3 in a series of migration inhibition tests (Table 2). Significant activity could be found for both ($P < 0.005$). The majority of the GT TFLA seemed to elute in fraction 3, the majority of the OA TFLA in fraction 2.

TABLE 1

ABILITY OF DEAE-CELLULOSE FRACTIONS TO LEAD TO GT-DEPENDENT MIGRATION INHIBITION

DEAE-cellulose fraction[a]	Migration area, average ± SD[b]		Percentage inhibition[c]	
	No antigen	GT antigen	Optimal dilution	Other dilution
No TFLA	203 ± 20	205 ± 15	−1	
2, 10^{18} dilution	221 ± 38	206 ± 24	7	6
3, 10^{19} dilution	215 ± 9	167 ± 14[d]	22	5
4, 10^{20} dilution	228 ± 17	216 ± 14	5	3
5, 10^{18} dilution	209 ± 20	148 ± 17[d]	29	12
6, 10^{19} dilution	188 ± 17	181 ± 17	4	Not tested

[a] TFLA were prepared through the DEAE-cellulose chromatography step as described under Materials and Methods. See also Fig. 1.

[b] The migration inhibition test was performed as described under Materials and Methods. In this experiment, TFLA were tested at 10^{18} and 10^{19} dilutions. Data (four replicates) are shown for the dilution that led to optimal inhibition.

[c] Percentage inhibition is shown for the dilution that was optimal, and for the other dilution tested.

[d] Smaller migration area than both no antigen and no TFLA controls, $P < 0.01$ (unpaired, one-tailed t test).

Specificity

The specificity of OA fraction 2 and GT fraction 3 was analyzed in a series of consecutive experiments (Table 3). Ovalbumin fraction 2 led to 23.9% inhibition (compared to a control of −3.2%) in the presence of OA, but to no significant inhibition in the presence of GT. GT fraction 3 led to inhibition in the presence of both GT and OA, and the responses were not significantly different. This result was not surprising in light of the possible cross-reaction in animals actively immunized to GT (see Donor Immune Responses).

Guinea pigs immunized to OA did respond to OA to a greater extent than did those immunized to GT. The response by cells treated with OA TFLA and OA was significantly greater than that by cells treated with GT TFLA and OA (Table 3, $P < 0.01$). The responses by cells treated with GT TFLA and GT, and OA TFLA and GT, were compared directly in two experiments. In those experiments, the responses by the former were greater by 29 and 40% inhibition. In the pooled, unpaired experiments, the inhibition by cells treated with GT TFLA and GT (21.8%) was greater than that by cells treated with OA TFLA and GT (8.7%, $P < 0.025$, unpaired, one-tailed t test).

Lack of Antigen in TFLA Preparations

It is possible that the activity of the TFLA resides in a superantigenic fragment. To investigate this possibility, OA and GT were radiolabeled with sodium [^{125}I]iodide guinea pigs were immunized with radiolabeled antigens, and TFLA were prepared from these guinea pigs. Fractions from DEAE-cellulose were concentrated by lyophilization and count rates were determined (Table 4). In three preparations, no counts significantly elevated above background could be detected. The few counts found in the immune cell lysates were either pelleted at

EXHIBIT A2

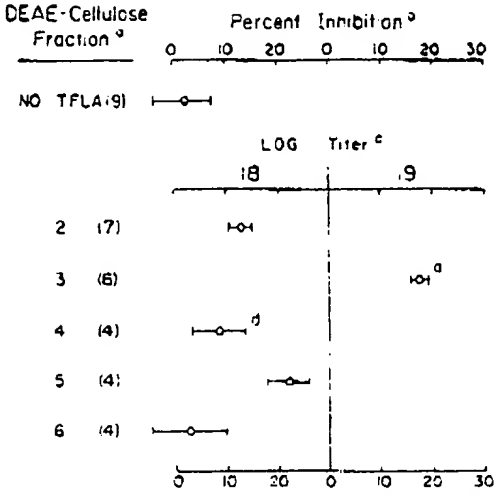

FIG. 2. Activity and titer of GT DEAE-cellulose fractions. (a) TFLAs were purified through the DEAE-cellulose chromatography step as described under Materials and Methods. See also Fig. 1. Number of migration inhibition experiments is shown in parentheses. (b) Migration inhibition tests in the presence of GT were performed as described under Materials and Methods. TFLAs were tested at 10^{18} and 10^{19} dilutions. Mean optimal percentage inhibitions are presented as geometric means with standard error bars. Each fraction was analyzed independently. (c) This is the log dilution, which, in most experiments, led to antigen-dependent migration inhibition. Fractions and the number of experiments in which the cited dilution was optimal/total are: fraction 2, 6/7; fraction 3, 5/8; fraction 4, 3/4; fraction 5, 4/4; fraction 6, 2/4. (d) Greater inhibition than no TFLA control, $P < 0.05$ (unpaired, one-tailed t test).

700*g* or excluded from Sephadex G-25. All three TFLA preparations were active in the migration inhibition test, tests of these factors comprising much of the data of Tables 2 and 3. Also two of three of the preparations were shown to have nmol amounts of primary amine (Table 4).

TABLE 2
ELUTION OF TFLAS FROM DEAE-CELLULOSE

TFLA donor immunization	*In vitro* antigen	Percentage inhibition, geometric mean ± SE[a]		
		No TFLA	DEAE-cellulose fraction 2	DEAE-cellulose fraction 3
OA	OA	−5.8 ± 4.1	19.0 ± 5.1[b]	5.3 ± 9.9
GT	GT	0.8 ± 3.8	8.6 ± 2.9	18.6 ± 3.3[b]

[a] This is a summary of experiments like that presented in Table 1. Each DEAE-cellulose fraction was tested at two or three dilutions in the migration inhibition test as described under Materials and Methods. Various column fractions were compared in a similar fashion to the analysis of specificity. Results for all fractions were reported at the same dilution, that dilution leading to significant inhibition at the highest titer. If fraction A led to 22% inhibition at a 10^{19} dilution and 8% at 10^{18}, and fraction B led to −8% at 10^{19} and 13% at 10^{18}, then the results were reported for that experiment at a 10^{18} dilution. We feel the 10^{19} dilution most accurately reflects the distribution of activity, and that the 13% inhibition in fraction B, if real, represents spillover from fraction A. Ovalbumin TFLAs were tested in 6 experiments, GT TFLA in 11 experiments.

[b] Significant inhibition compared to no TFLA controls, $P < 0.005$ (unpaired, one-tailed t test).

TABLE 3
SPECIFICITY OF DEAE-CELLULOSE-ISOLATED TFLA

TFLA[a]	Percentage inhibition, geometric mean ± SE[b]	
	OA antigen	GT antigen
None	−3.2 ± 4.6 (10)	14.3 ± 1.9 (5)
OA, DEAE-cellulose fraction 2	23.9 ± 5.3 (10)[c]	8.7 ± 9.5 (5)
None	−9.8 ± 6.0 (8)	5.7 ± 3.9 (15)
GT, DEAE-cellulose fraction 3	5.2 ± 2.2 (8)[d]	21.8 ± 1.8 (17)[e]

[a] TFLA were purified through the DEAE-cellulose chromatography step as described under Materials and Methods.

[b] The migration inhibition test was performed as described under Materials and Methods. Number of determinations are shown in parentheses.

[c] Inhibition in the presence of antigen and TFLA was compared to that in the presence of antigen alone by unpaired, one-tailed t tests, $P < 0.005$. Inhibition for the specific antigen (OA in this case) was greater than inhibition for the nonspecific antigen (GT) in a paired, one-tailed t test, $P < 0.0005$. In the first three experiments of this series, GT was used at 50 μg/ml. This concentration proved to be nonspecifically inhibitory, leading to some migration inhibition in nonimmune animals (in the absence of either OA or GT TFLA). Inhibition of cells treated with OA TFLA and OA antigen was also compared to that of cells treated with GT TFLA and OA antigen in five experiments by a paired, one-tailed t test. $P < 0.01$.

[d] Significant inhibition when compared to no TFLA control, $P < 0.025$.

[e] Significant inhibition when compared to no TFLA control, $P < 0.0005$. Not significantly greater inhibition than for OA antigen with GT TFLA.

Analysis for 12 amino acids by gas chromatography revealed quantities of tyrosine, valine, leucine, aspartic acid, lysine, glycine, serine, glutamic acid, alanine, threonine, and proline in one GT TFLA preparation. All amino acids, except glycine, were present at approximately the 5-μmol level. Glycine was elevated 5- to 10-fold.

DISCUSSION

The work described herein involved partial purification of factors from guinea pigs leukocytes, documentation of the activity of those factors in the migration inhibition test, and evaluation of those factors for the presence of antigen fragments. The OA and GT factors were purified as previously described for OA and bovine γ globulin TFLA (11).

Biological Activity

Nonimmune peritoneal exudate cells, when incubated with both specific antigen and partially purified TFLA from OA- or GT-immunized guinea pigs are inhibited from migrating. As noted previously (10, 16) the TFLA activity is restricted to dilutions near 10^{19}. Using the value of 10^5 nmol amino acid per one animal preparation (GT TFLA, Table 3), one can calculate that each migration inhibition test would receive about 1 molecule of amino acid (10^5 nmol/10 ml × 6 × 10^{14} molecules/nmol × 10^{19} dilution). This sort of calculation implies an error in dilution, in assessing biological activity, or in chemical analysis.

EXHIBIT A?

TABLE 4

PREPARATION OF TFLA FROM DONORS IMMUNIZED WITH RADIOLABELED ANTIGEN

Immunizing antigen	Specific activity (cpm/μg)	Final product	Net cpm	Primary amine (nmol)	Amino acids (nmol)
^{125}I OA, 6.58×10^8 cpm	5.5×10^4	DEAE-cellulose fraction 2	0 ± 11^a	12[b]	Not done
^{125}I GT 7.68×10^7 cpm	7.7×10^4	DEAE-cellulose fraction 3	14 ± 20^a	24[b]	ca. 10^5
^{125}I GT 13.7×10^7 cpm	1.1×10^5	DEAE-cellulose fraction 3	5 ± 19^c	Not done	Not done

Note. Antigens were radiolabeled, TFLAs were prepared, primary amines were determined by fluorescamine reactivity, and amino acids were determined by gas chromatography as described under Materials and Methods.

[a] Background, 227 ± 9. Count rates are averages ± SD of five 1-min determinations.

[b] Control ammonium bicarbonate samples were 6 nmoles and were subtracted from values for primary amines.

[c] Background, 191 ± 5.

The dilution is unlikely to error by as much as 10-fold, although in other systems apparent dilution errors of 10^9-fold have been observed (18). The biological activity seems to be real. It is consistently observed in blindly evaluated experiments. Its elution position upon fractionation is consistent. The phenomenon has been reproduced by one of us in two different laboratories, and by an independent laboratory (16).

The amino acid content of TFLA has been approximated by both gas chromatography and by the fluorescamine reaction. The gc amino acid analysis involved hydrolysis of the TFLA sample, derivatization of any amino acids present, and separation of individual amino acids by gc. The fluorescamine assay involved reaction of a nonfluorescent reagent with amino acids, and other primary amines, in the TFLA to yield a fluorescent product. The value for amino acid content as determined by gc is about 10^4 higher than previously determined by the fluorescamine reaction ((11) and Table 3). The fluorescamine assay is probably in error due to an inner filter effect. At very high concentrations of fluorescent moiety (fluorescamine–amino acids in this case) all fluorescence takes place at the face of the cuvette, and is not detected (19). Considering the amino acid content as determined by gc, and the extinction coefficient of fluorescamine–amino acid reaction products (20), it is likely that this sort of error took place.

The gc analysis was semiquantitative, and could error by 3- to 10-fold. Experiments specifically designed to approach the potency problem will have to be completed to obtain an accurate estimate of the titer of TFLA.

The reactivities of the TFLA donors and recipients are related. Nonimmune peritoneal exudate cells incubated with DEAE-cellulose-purified OA TFLA and OA are inhibited from migrating, those with OA TFLA and GT are not (Table 3). On the other hand, the migration inhibition by cells incubated with GT TFLA and GT is highly significant.

Cells incubated with GT TFLA and OA also appear to be significantly inhibited from migrating. If real (putting aside the interpretation of a 5.2% inhibition com-

pared to a control of −9.8%), this migration inhibition could represent cross-reactivity between OA and GT. Guinea pigs immunized to GT seem to respond to OA by slight migration inhibition (see Donor Immune Responses), while nonimmune cells do not (see No TFLA controls, Tables 2 and 3). Thus, GT and OA may cross-react in one direction, as has been shown for GT and glutamic acid–alanine–tyrosine copolymers in another system (21).

The evidence for specificity is strengthened by the fact that cells incubated with OA TFLA and OA are inhibited more than cells incubated with GT TFLA and OA (Table 3). Also, in the experiments available, cells incubated with GT TFLA and GT seem to be inhibited more than cells incubated with OA TFLA and GT. In total, the data indicate specificity for TFLA purified through the DEAE-cellulose chromatography step. Others have also tested transfer factor-like activities in leukocyte dialysates by migration inhibition (4, 12–14). This activity, when tested, seems to be specific for donor immunities (4, 12).

If OA and GT are to be functionally specific, they must be structurally distinct. There is some suggestion that OA and GT TFLA are separable on DEAE-cellulose columns; OA TFLA seems to elute in fraction 2 and GT TFLA in fraction 3 (Fig. 1 and Table 2). This separation has not been proven.

Superantigenicity

Several aspects of the activity and molecular nature of TFLA could be explained if TFLA were a superantigen. If antigen is a major part of GT TFLA, then the factor must include either tyrosine or glutamic acid residues, or both. Both GT and OA were labeled with ^{125}I, donor guinea pigs were immunized with these radiolabeled antigens, and TFLA were prepared through the DEAE-cellulose chromatography step. No significant amount of radioactivity could be found in the purified TFLA or in the Sephadex G-25 fraction known to be active in the migration inhibition test (10, 11, 16). For the first GT factor tested, the maximum counts possible in fraction 3 would be about 54 (mean + 2 SD). This corresponds to 7×10^{-4} μg GT. However, the GT DEAE-cellulose fraction 3 was found to contain ca. 10^4 μg amino acid by gc. If TFLA is to be superantigen, it must be less than 0.000001% of the DEAE-cellulose-active fraction.

Alternatively, TFLA could be superantigen, and tyrosine residues might be excluded (for unknown reasons). Thus, for GT TFLA all antigenic residues must be glutamic acid. Nevertheless, amino acid analysis of the active GT TFLA revealed both glutamic acid and tyrosine, at approximately the same level as each other and as eight other amino acids.

Baram and Mosko (8) and Burger *et al.* (9) have shown that the ability of transfer factor to transfer dermal reactivity to a given antigen is not lost when the transfer factor is given the opportunity to bind to antisera against that antigen. This approach does not require purified transfer factor, but does assume that antigen fragments in the transfer factor can bind to antibodies. The approach used in this study allows detection of any GT fragment containing tyrosine, but assumes some purity for the TFLA (albeit much less than 1%). In this sense, the studies of Baram, Burger, and their colleagues and this study complement each other.

It seems that superantigenicity cannot explain the activity of TFLA. One should be cautious when extending the results of this investigation to human transfer factor active *in vivo*. Whereas transfer factor and TFLA are structurally similar (11, 2–4), and the tests for the two are related, no direct relationship has been established between TFLA and *in vivo* transfers of cellular immunity.

ACKNOWLEDGMENTS

The authors thank Patricia Finke and Carolyn Barney for technical assistance, Robert Shoemake for expert animal care, Jane Pfenning for typing the manuscript, and W. Frick and D. Daves for performing and interpreting the gas chromatographic analysis of amino acids.

REFERENCES

1. Lawrence, H. S., *Harvey Lect.* 68, 239, 1974.
2. Burger, D. R., Vandenbark, A. A., Dunnick, W., Kraybill, W., Daves, G. D., and Vetto, R. M., *J. Immunol.* 122, 1091, 1979.
3. Rifkind, D., Frey, J. A., Petersen, E. A., and Dinowitz, M., *Infect. Immun.* 16, 258. 1977.
4. Paddock, G. V., Wilson, G. B., Fudenberg, H. H., Wang, A.-C., and Lovins, R. E., *In* "Immune Regulators in Transfer Factor" (A. Kahn, C. H. Kirkpatrick, and N. O. Hill, Eds.), Academic Press, New York, in press.
5. Fishman, M., and Adler, F. L., *J. Exp. Med.* 117, 595, 1963.
6. Askonas, B. A., and Rhodes, J. M., *Nature (London)* 205, 470, 1965.
7. Friedman, H. P., Stavitsky, A. B., and Soloman, J. M., *Science* 149, 1106, 1965.
8. Baram, P., and Mosko, M., *Immunology* 8, 461, 1965.
9. Burger, D. R., Malley, A. A., and Vetto, R. M., *Science* 175, 1473, 1972.
10. Dunnick, W., and Bach, F. H., *Proc. Nat. Acad. Sci. USA* 72, 4573, 1975.
11. Dunnick, W., and Bach, F. H., *J. Immunol.* 118, 1944, 1977.
12. Brandes, L., and Goldberg, G. J., *Cancer Res.* 34, 3095, 1974.
13. Salaman, M. R., *Immunology* 26, 1069, 1974.
14. Baram, P., and Condoulis, W., *Transplant. Proc.* 6, 209, 1974.
15. Frick, W., Chang, D., Folkers, K., and Daves, G. D., *Anal. Chem.* 49, 1241, 1977.
16. Whitacre, C., and Paterson, P. Y., *Clin. Immunol. Immunopathol.* 13, 287, 1979.
17. Bluestein, H. G., Green, I., and Benacerraf, B., *J. Exp. Med.* 134, 458, 1971.
18. Pearce, F. L., Banthorpe, L. V., Cook, J. M., and Vernon, C. A., *Eur. J. Biochem.* 32, 569, 1973.
19. Cantor, C. R., and Tao, T., *In* "Procedures in Nucleic Acid Research, (G. L. Cantoni and D. R. Davies, Eds.), p. 56, Harper & Row, New York, 1971.
20. Weigle, M., DeBernardo, S. L., Tengi, J. P., and Leimgruber, W., *J. Amer. Chem. Soc.* 94, 5927, 1972.
21. Waltenbaugh, C., Debre, P., and Benacerraf, B., *J. Immunol.* 117, 1603, 1976.